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Symbioimine and neosymbioimine, amphoteric iminium metabolites from the symbiotic marine dinoflagellate *Symbiodinium* sp.☆

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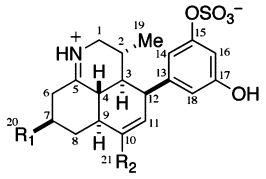
Abstract—Two amphoteric iminium metabolites, symbioimine (**1**) and neosymbioimine (**2**), were isolated from a cultivated symbiotic marine dinoflagellate *Symbiodinium* sp. Compounds **1** and **2** have a characteristic 6,6,6-tricyclic iminium ring structure and an aryl sulfate moiety. The plausible biogenetic pathway of **1** and **2** can be explained by an intramolecular Diels–Alder reaction followed by imine cyclization. Symbioimine (**1**) inhibited the differentiation of RAW264 cells into osteoclasts (EC_{50} = 44 μM), and significantly inhibited cyclooxygenase-2 activity at 10 μM. Thus, symbioimine is a potent anti-resorptive and anti-inflammatory drug.

1. Introduction

A considerable number of fascinating natural products with unique chemical structures and biological activities have been found in marine organisms. However, the true origin or progenitors of these metabolites are not entirely clear.[1–3] The possible primary producers of secondary metabolites have been suggested to be microalgae, bacteria, and fungi, and they are carried at through symbiosis, association, a food chain, and other forms of nutrient dependency. We have been interested in bioactive metabolites produced by marine symbiotic microorganisms. The symbiotic marine dinoflagellate *Symbiodinium* sp., which is a type of zooxanthellae, is found in a wide range of marine invertebrates[4–6] and produces several super-carbon-chain compounds,[7] such as zooxanthellatoxins[8–10] and zooxanthellamides.[11,12] It has also been suggested that bioactive alkaloidal metabolites of cyanobacteria may help to inhibit predation by marine herbivores, such as fish and sea urchins in the ecosystem.[13] Although the true ecological role of these compounds in the symbiotic dinoflagellate is unknown, they may serve as a defense material which prevents digestion of their host animal. We previously reported a unique amphoteric iminium compound, symbioimine (**1**), from a culture of the dinoflagellate *Symbiodinium* sp.[14,15] In our continuing search for biologically active compounds, a congener of **1**, named neosymbioimine (**2**), has been isolated. In this paper, we describe the isolation, structure elucidation, and biological activities of these compounds.



1 : $R_1 = R_2 = H$ (Symbioimine)
2 : $R_1 = R_2 = Me$ (Neosymbioimine)

2. Results and discussion

2.1. Isolation of symbioimine and neosymbioimine

The cultivated dinoflagellate (36 g), isolated from the marine acoel flatworm *Amphiscolops* sp., was extracted with 80% aqueous ethanol. The concentrated extract was partitioned with ethyl acetate and water, and the

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aqueous layer was chromatographed on TSK G-3000S polystyrene gel and DEAE–Sephadex. Final purification was achieved by reverse-phase HPLC to give symbioimine (**1**) (5.7 mg).[14] We found that a trace amount of the congener of **1** was present in the fraction eluted after **1** on HPLC. Finally, 4.2 mg of the minor component, named neosymbioimine (**2**), was successfully isolated from 112 g of the cultivated dinoflagellate.

2.2. Structure of symbioimine

The molecular formula of **1** was found to be $C_{19}H_{23}NO_5S$ [$(M + H)^+$, *m/z* 378.1368, Δ −0.7 mmu; $(M-H)^-$, *m/z* 376.1213, Δ −0.6 mmu] by HRESIMS. Negative ESIMS also showed a characteristic fragment ion [$(M-SO_3H)^-$, *m/z* 296] that suggested the presence of a sulfate moiety. A detailed analysis of the ^1H, ^{13}C NMR, COSY, HMQC, and HMBC spectra in DMSO-d_6 showed that **1** contained one methyl group, four methylenes, 10 methines, four quaternary carbons, and two protons on heteroatoms (δ_{NH} 12.82, δ_{OH} 9.35) (Table 1). In addition to supporting the presence of hydroxyl groups (3450 cm^{-1}), the IR spectrum of **1** showed absorption bands for iminium (1690 cm^{-1}) and sulfate (1240, 1140, and 1050 cm^{-1}) groups. The ^{13}C NMR signal at 188.0 (C-5) implied the presence of an iminium functionality in this water-soluble amphoteric compound.

A detailed analysis of the COSY spectrum of **1** suggested partial carbon–carbon connectivities: C-1–C-12 except for C-5 and a methyl group (C-19). Homoallylic coupling between H-1/H-4 and HMBC correlations between H-1/C-5, H-4/C-5, and H-6_b/C-5 were observed, which suggested that C-5 could be assigned to be the carbon of the imine group. Moreover, the allylic coupling between H-9/H-11 and the HMBC correlation between H-10/C-8 indicated that **1** possessed a 6,6,6-tricyclic ring system that included C-1–C-12 and a nitrogen atom.

The aromatic ring structure of **1** was established by HMBC correlations H-14/C-15 and C-18, H-16/C-15 and C-17, H-18/C-14 and C-17. Three aromatic protons could be placed on a 3,5-dioxygenated benzene based on the magnitudes of *meta*-coupling ($J_{14,16} = J_{14,18} = J_{16,18}$ = 1.8 Hz) and the ^{13}C NMR chemical shifts (δ_{C-15} 154.1 and δ_{C-17} 157.2). The HMBC correlations between O*H*/C-16 and C-18 confirmed that the hydroxyl group was directly connected to the aryl carbon (C-17). Due to the possible asymmetry of the aryl moiety (H-14 and H-18, C-14 and C-18, and C-15 and C-17), the remaining oxygenated aryl carbon (C-15) may be linked to a sulfate group. The HMBC correlations H-3/C-13, H-12/C-13 and C-14, H-14/C-12, and H-18/C-12 suggested that the aryl carbon (C-13) was linked to the methine carbon (C-12). Thus, symbioimine (**1**) was confirmed to be a 6,6,6-tricyclic iminium ring compound possessing an aryl sulfate moiety.

The relative stereochemistry of **1** was deduced as follows. The large magnitudes of $J_{1a,2}$ = 12.0 Hz, $J_{2,3}$ = 11.1 Hz, $J_{3,4}$ = 11.1 Hz, $J_{4,9}$ = 11.9 Hz, $J_{7a,8a}$ = 12.9 Hz, and $J_{8a,9}$ = 12.9 Hz suggested that all these seven protons, H-1a, H-2, H-3, H-4, H-7a, H-8a, and H-9a, were oriented in anti-arrangements with respect to the tricyclic ring (Fig. 1). Thus, the three six-membered rings may show *trans* ring fusion with each other and the methyl group (C-19) may be oriented in a pseudo-equatorial conformation with respect to the six-membered iminium ring with a twist-boat conformation. NOEs

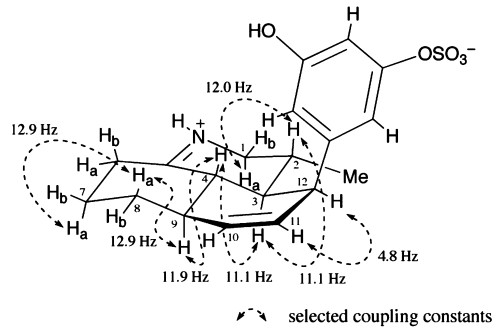


Figure 1. Relative stereochemistry of symbioimine (**1**).

Table 1. ^1H and ^{13}C NMR data of symbioimine (**1**) in DMSO-d_6

Atom	^{13}C[a]	^1H[b]	Atom	^{13}C[a]	^1H[b]
1a	50.0 t	3.14 dd (12.0, 14.3)	10	130.4 d	5.79 br d (9.6)
1b		3.58 dd (4.3, 14.3)	11	129.5 d	5.67 ddd (2.7, 4.8, 9.6)
2	26.2 d	1.25 dddq (4.3, 11.1, 12.0, 6.2)	12	41.7 d	3.65 t (4.8)
3	40.8 d	1.77 dt (4.8, 11.1)	13	139.8 s	
4	40.1 d	2.55 dd (11.1,11.9)	14	112.8 d	6.53 t (1.8)
5	188.0 s		15	154.1 s	
6a	33.8 t	2.65[c] m	16	105.8 d	6.62 t (1.8)
6b		2.68[c] m	17	157.2 s	
7a	24.4 t	1.67 m	18	111.7 d	6.40 t (1.8)
7b		2.03 m	19	15.6 q	1.05 d (6.2) 3H
8a	29.8 t	1.50 dq (4.2, 12.9)	NH		12.82 br s
8b		1.94 br dd (2.5, 12.9)	OH		9.35 s
9	41.4 d	2.41 br ddd (2.7, 11.9, 12.9)			

[a] Recorded at 201 MHz. Multiplicity was based on HMQC spectrum.
[b] Recorded at 800 MHz. Coupling constants (Hz) are in parenthesis. Higher-field methylene protons are labeled 'a' and lower-field signals are 'b.'
[c] Signals may be interchanged.

were observed for H-4/H-14 and H-4/H-18, suggesting that the aryl moiety may be oriented in a pseudo-axial conformation with respect to the cyclohexene ring with a twist-boat conformation. Finally, the stereostructure of **1** was confirmed by X-ray crystallographic analysis.[14] The absolute stereochemistry of **1** was confirmed to be 2*R*, 3*R*, 4*S*, 9*R*, 12*S*, from the value of the Flack parameter 0.03(13).

2.3. Structure of neosymbioimine

The molecular formula of neosymbioimine (**2**) was found to be $C_{21}H_{27}NO_5S$ [$(M-H)^-$ (*m*/*z* 404.1540, Δ + 0.9 mmu) by HRESIMS, which reflects a 28 MS unit (C_2H_4) increase compared with that of **1**. Negative ESIMS showed a characteristic fragment ion [$(M-SO_3H)^-$, *m*/*z* 324] suggesting the presence of a sulfate moiety, as with **1**. The ^1H and ^{13}C NMR spectra of **2** are summarized in Table 2. The presence of two doublet methyl groups and one vinyl methyl group in **2** was established from these data. The ^{13}C NMR signal at 189.3 (C-5) implied the presence of an iminium moiety in **2**. A detailed analysis of the COSY spectrum of **2** led to partial carbon–carbon connectivities: C1–C12 except for C-5 and C-10, and two methyl groups (C-19, 20) (Fig. 2). Homoallylic coupling between H-1a/H-4 and HMBC correlations between H-1/C-5, H-4/C-5, and H-6/C-5 were observed, which suggested a six-membered cyclic imine moiety. Notably, a gradual disappearance of the proton signals H-6a and 6b (δ_H 2.25, 2.56) in CD_3OD due to deuterium exchange also suggested that this proton is adjacent to the imine carbonyl moiety.[16] The allylic coupling between H-11/H_3-21 and the HMBC correlations between H-9/C-10 and H-11/C-21. H_3-21/C-9, C-10, and C-11 confirmed that the three carbons C-9, C-11, and C-21 were directly connected to a quaternary carbon (C-10). Thus, a 6,6,6-tricyclic ring system of **2** that included three methyl groups and a nitrogen atom was established.

The aromatic ring structure of **2** was established by HMBC correlations H-14/C-18, H-16/C-17, and H-18/C-14 and C-17. Three aromatic protons could be placed on a 3,5-dioxygenated benzene based on the magnitudes of *meta*-coupling ($J_{14,16} = J_{14,18} = J_{16,18}$ = 1.9 Hz) and the ^{13}C NMR chemical shifts ($\delta_{C\text{-}15}$ 158.8 and $\delta_{C\text{-}17}$ 154.4). Due to the possible asymmetry of the aryl moiety (H-14 and H-18, C-14 and C-18, and C-15 and C-17), the two oxygenated aryl carbons (C-15 and C-17) may be linked to a sulfate group and a hydroxyl group, respectively, as in **1**. HMBC correlations H-3/C-13, H-12/C-13, H-14/C-12, and H-18/C-12 suggested that the aryl carbon (C-13) was linked to the methine carbon (C-12). Thus, neosymbioimine (**2**) was confirmed to be a 6,6,6-tricyclic iminium ring compound possessing an aryl sulfate moiety and three methyl groups, as shown in Figure 2.

Considering the chemical shifts and the coupling constants in the ^1H and ^{13}C NMR spectra, it was proposed that the stereochemistries of symbioimine (**1**) and neosymbioimine (**2**) may be superimposed on each other. The relative stereochemistry of **2** was deduced as follows. The large magnitudes of $J_{1a,2}$ = 11.2 Hz, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 11.0 Hz, $J_{4,9}$ = 12.4 Hz, $J_{7,8a}$ = 12.4 Hz, and $J_{8a,9}$ = 12.4 Hz suggested that all these seven protons, H-1a, H-2, H-3, H-4, H-7, H-8a, and H-9a, were

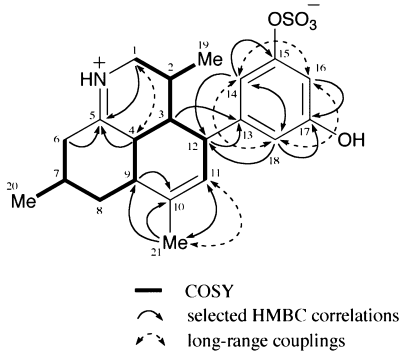


Figure 2. Structure of neosymbioimine (**2**), based on 2D NMR correlations.

Table 2. ^1H and ^{13}C NMR data of neosymbioimine (**2**) in CD_3OD

Atom	^{13}C[a]	^1H[b]	Atom	^{13}C[a]	^1H[b]
1a	52.6 t	3.14 br dd (11.2, 15.8)	10	137.6 s	
1b		3.55 dd (3.9, 15.8)	11	126.4 d	5.52 br d (4.8)
2	27.9 d	1.33 m	12	43.3 d	3.70 t (4.8)
3	43.6 d	1.77 dt (4.8, 11.1)	13	142.3 s	
4	41.6 d	2.62 br dd (11.1, 12.4)	14	114.9[d] d	6.58[d] t (1.9)
5	189.3 s		15	158.8[e] s	
6a	42.8 t	2.25[c] m	16	108.5 d	6.60 t (1.9)
6b		2.56[c] m	17	154.4[e] s	
7	34.0 d	1.91 m	18	115.7[d] d	6.79[d] t (1.9)
8a	37.5 t	1.28 q (12.4)	19	16.3 q	1.15 d (5.9) 3H
8b		2.25 td (3.4, 12.4)	20	22.1 q	1.12 d (6.5) 3H
9	46.1 d	2.32 dt (3.4, 12.4)	21	21.1 q	1.83 br s 3H

[a] Recorded at 150 MHz. Multiplicity was based on HMQC spectrum.
[b] Recorded at 800 MHz. Coupling constants (Hz) are in parenthesis. Higher-field methylene protons are labeled "a" and lower-field signals are "b."
[c] Signals may be interchanged.
[d] Signals may be interchanged.
[e] Signals may be interchanged.

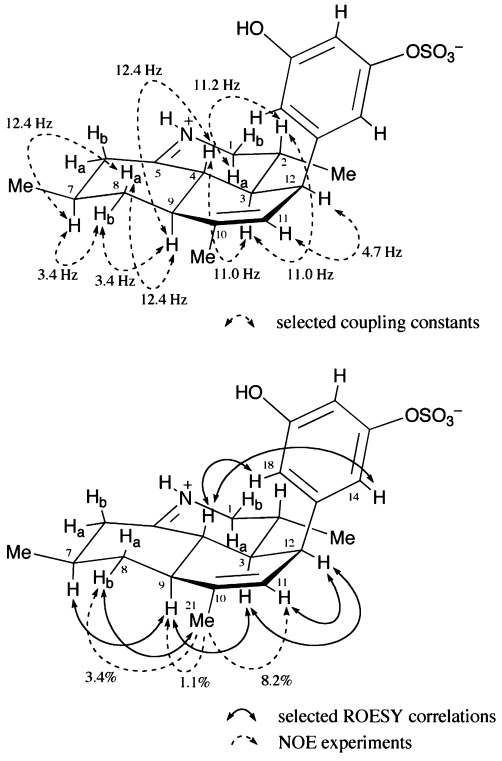
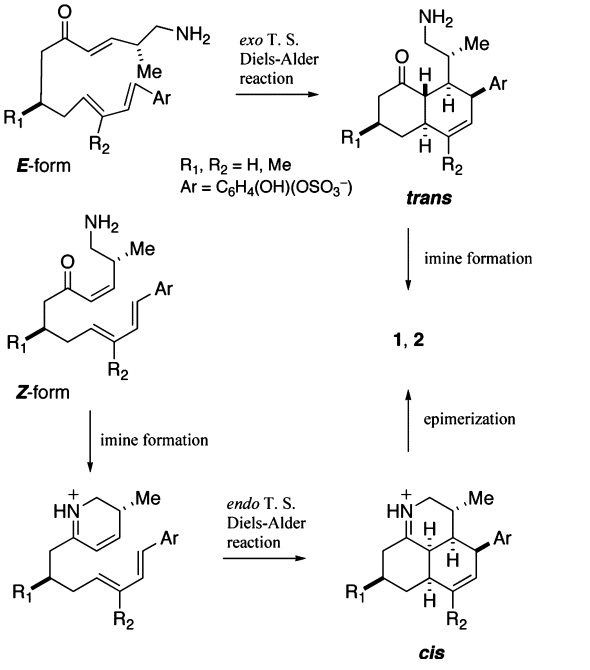


Figure 3. Relative stereochemistry of neosymbioimine (**2**).



Scheme 1. Plausible biogenetic pathway for the carbon framework of symbioimine (**1**) and neosymbioimine (**2**).

oriented in anti-arrangements with respect to the tricyclic ring (Fig. 3). In NOE experiments (800 MHz) on **2**, NOEs were observed for H-3/H-9, H-3/H-12, H-7/H-9, H-8b/H_3-21, H-9/H_3-21, H-11/H_3-21, and H-11/H-12. These results suggested that these three six-membered rings may show *trans* ring fusion with each other and that the two methyl groups (C-19 and C-20) may be oriented in a pseudo-equatorial conformation with respect to the six-membered iminium ring with a twist-boat conformation, as in **1**. NOEs were also observed for H-4/H-14 and H-4/H-18, suggesting that the aryl moiety may be oriented in a pseudo-axial conformation with respect to the cyclohexene ring with a twist-boat conformation. Since symbioimine (**1**) and neosymbioimine (**2**) are biosynthesized by the same dinoflagellate, their absolute stereochemistries are suggested to be identical. Thus, the absolute stereochemistry of **2** was established to be 2*R*, 3*R*, 4*S*, 7*R*, 9*R*, 12*S*.

2.4. Biogenesis of symbioimine and neosymbioimine

The unique structures of **1** and **2**, including a 6,6,6-tricyclic iminium ring, can be explained by the plausible biogenetic pathway shown in Scheme 1. An intramolecular *exo* transition state Diels–Alder reaction followed by imine cyclization could form the carbon framework of **1** and **2** stereospecifically, as in the case of pinnatoxins.[17] Since the 6,6,6-tricyclic iminium ring moiety of neosymbioimine (**2**) is composed of 15 carbons including three methyl groups, a sesquiterpene biosynthesis pathway can be considered. However, due to its inconsistency with the products in the normal sequential head-to-tail condensation of two molecules of isopentenyl diphosphate with dimethylallyl diphosphate, we proposed the polyketide synthesis pathway for construction of the characteristic C1–C12 moiety in **1** and **2**. Recently, a synthesis of (+)-dihydrocompactin was reported.[18] It was suggested that cyclic oxocarbenium ion intermediate was formed and acted as a plausible dienophile in the *endo*-transition state Diels–Alder reaction to provide *cis*-octalone stereospecifically, which was epimerized to give *trans*-octalone. From this point of view, it can be also considered that the tricyclic ring in **1** and **2** are biosynthesized by a cyclic imine formation, *endo*-transition state Diels–Alder reaction followed by an epimerization. Various marine metabolites with an imine moiety have been described, that is pinnatoxins,[17,19–21] pteriatoxins,[22] spirolides,[23] gymnodimine,[24] prorocentrolide,[25] and spiro-prorocentrimine,[26] but no natural compounds with a tricyclic iminium ring structure, such as in **1** and **2**, have been reported in either terrestrial or marine organisms. Studies of the biosynthetic pathways of **1** and **2** using isotope-labeled precursor incorporation studies are in progress.

2.5. Biological activities of symbioimine

Symbioimine (**1**) inhibited osteoclastogenesis in the murine monocytic cell line RAW264, which can differentiate into osteoclasts following treatment with receptor activator of nuclear factor-κB ligand (RANKL) (EC_{50} = 44 μg/mL) (Fig. 4).[14] RANKL induces the formation of osteoclast-like multinucleated cells in cultures of bone marrow cells. Symbioimine (**1**) inhibited an increase in sRANKL-induced tartrate-resistant acid phosphatase (TRAP) activity in preosteoclast cells.[27–29] Meanwhile, it did not affect cell viability even at 100 μg/mL. Thus, symbioimine (**1**) is a potential antiresorptive drug for the prevention and treatment of osteoporosis in postmenopausal women.

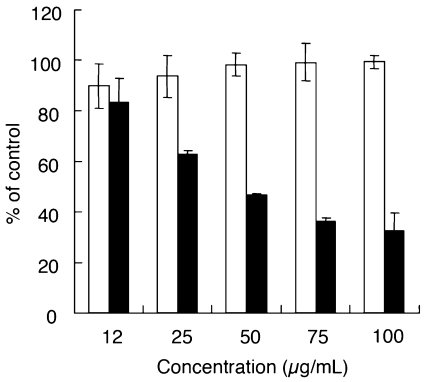


Figure 4. The effects of symbioimine (**1**) on formation of osteoclast-like cell (filled columns) and cell viability (open columns).

Symbioimine (**1**) also significantly inhibited cyclooxygenase-2 (COX-2) activity (32%) at 10 μM. Meanwhile, its ability to inhibit COX-1 was not very strong (5%) at 10 μM. The overexpression of COX-2 has been observed in many kinds of tumors, and its role in carcinogenesis and angiogenesis has been extensively investigated.[30–32] Several COX-2 selective inhibitors, such as rofecoxib, celecoxib, and sulindac, have been developed. Due to its moderate subtype specificity, symbioimine (**1**) may be useful for the development of new nonsteroid anti-inflammatory drugs (NSAID) to treat COX-associated diseases, such as inflammatory diseases and cancer.

3. Conclusion

In conclusion, symbioimine (**1**) and neosymbioimine (**2**) were isolated from the culture of the symbiotic marine dinoflagellate *Symbiodinium* sp., and were determined to be novel amphoteric cyclic iminium metabolites by NMR, HRMS, and X-ray crystallographic analyses. It was also shown that symbioimine significantly inhibited osteoclastogenesis and COX-2 activity. Further studies on the biological activities and biosynthesis of symbioimine and neosymbioimine are in progress.

4. Experimental

4.1. General

Melting points were determined using a Yanaco MP-J3 hot-stage melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-1000 polarimeter. IR spectra were recorded on a JASCO FT/IR-230 spectrometer using a KBr pellet. NMR spectra were recorded on a JEOL JNM-ECA800 (800 MHz for ^{1}H and 201 MHz for ^{13}C) instrument. The chemical shifts were referred to the solvent peaks: δ_H 3.31 (residual $C\underline{H}D_2OD$) and δ_C 49.0 for CD_3OD, δ_H 2.49 (residual $C_2\underline{H}D_5OS$) and δ_C 39.5 for DMSO-d_6. High-resolution electrospray ionization mass spectra (HR-ESIMS) were obtained on a PE Biosystems QSTAR mass spectrometer. Fuji silysia silica gel BW-820MH and Nacalai Tesque Cosmosil 75C_{18}-OPN were used for column chromatography. Merck precoated silica gel 60 F_{254} plates were used for thin-layer chromatography (TLC).

4.2. Isolation of symbioimine (1) and neosymbioimine (2)

The dinoflagellate *Symbiodinium* sp. was separated from the interior cells of the marine acoel flatworm *Amphiscolops* sp. (three individuals), which was collected from the reef of Sesoko Island, Okinawa, Japan. The dinoflagellates were cultivated at 23 °C and 70% RH for 20 days in sterilized seawater medium enriched with 2% Provasoli's Ert-Schreiber (ES) supplement, under a 10 h/14 h light/dark cycle. The harvested cells (36 g wet weight from 80 L of culture) were extracted with 80% aqueous ethanol (300 mL) for three days. The extract was filtered, and the residue was boiled with 80% aqueous ethanol (300 mL) for 2 min and extracted at room temperature for one day. The combined extracts were concentrated, and the residue (4.5 g) was partitioned with ethyl acetate (3 × 300 mL) and water (300 mL). The aqueous layer was loaded on a TSK G-3000S polystyrene gel column (ϕ 30 × 50 mm, Tosoh Co., Osaka, Japan) and eluted with 0, 25, 50, 75, and 100% aqueous ethanol (150 mL each). The concentrated 50% aqueous ethanol fraction (105 mg) was loaded on a DEAE–Sephadex column (ϕ 17 × 170 mm, Amersham Bioscience), and eluted with 20 and 200 mM phosphate (pH 6.9), and 2 M NaCl (50 mL each). The concentrated 20 mM phosphate fraction (56 mg) was applied twice to a Develosil TMS UG-5 reversed-phase HPLC column (ϕ 20 × 250 mm, Nomura Chemical Co., Aichi, Japan). A linear gradient of 20%–40% aqueous acetonitrile was applied for 210 min at a flow rate of 5 mL/min, and was monitored at 215 nm, to give symbioimine (**1**) (5.7 mg, 0.016% based on wet wt, t_R = 32.0 min). Because only a small amount of neosymbioimine (**2**) was obtained, cultured dinoflagellate (112 g) was separated once more. An additional HPLC purification using the same conditions as above afforded **2** (4.2 mg, 0.0038% based on wet wt, t_R = 74.0 min).

4.2.1. Symbioimine (1). A colorless powder; m.p. 214–215 °C (dec.); $[\alpha]_D^{27}$+245° (*c* 0.10, DMSO); IR (KBr) 3450, 1690, 1610, 1515, 1450, 1260, 1240, 1140, 1050 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 378.1368 $(M+H)^+$ (calcd for $C_{19}H_{24}NO_5S$, Δ −0.7 mmu) and *m/z* 376.1213 $(M-H)^-$ (calcd for $C_{19}H_{22}NO_5S$, Δ −0.6 mmu).

4.2.2. Neosymbioimine (2). A colorless oil; $[\alpha]_D^{23}$+149° (*c* 0.10, MeOH); ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 404.1540 $(M-H)^-$ (calcd for $C_{21}H_{27}NO_5S$, Δ +0.9 mmu).

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